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Enzymatic High Digestion of Soybean Milk Residue (Okara)

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The objective of this study was to digest okara in high yield by food-processing enzymes. Autoclaving of okara was effective in increasing cellulase digestion for the primary cell wall, and the digestion was accelerated by the formation of single cells by stirring. Most of the residual okara after autoclaving and cellulase digestion was found to be the secondary cell walls compared with the cellulase-treated soybean single cells. The secondary cell wall was found to be composed of galacturonic acid, neutral sugars, and protein and was considered to be a complex of these compositions. Many celullolytic and proteolytic enzymes could not digest the secondary cell wall; however, it was found that two pectinases could digest the secondary cell wall. A series of digestions resulted in yields of 83−85% from the raw okara, and the final residues were identified as oil body complexes in the soybean cells and fiber-like organ between the cells.

KEYWORDS: Soybean residue; okara; cellulase; pectinase; cell wall; enzymatic digestion

INTRODUCTION

Soybean is an important food seed, which contains good proteins and oil, and many foods and/or foodstuffs are made from it. Okara is the filtered residue from which soybean milk is separated and is formed in large quantities along with the manufacture of soybean milk, tofu, and fried bean curd, especially in Asian areas (*1*). Recently, it has become a typical agricultural waste because the reuse of okara is very difficult, although many trials of its use have been done (*1*).

Our research was aimed at the solubilization of okara using a new approach to show the possibility of using okara, because solubilization is fundamentally a simple method to use and/or to study and to explore a new food function.

Okara comes from soybeans essentially; protein and oil from soybeans would remain, and the carbohydrates and fibers from the cells of the soybeans would also remain (*2*−*4*). There are many reports on the component analysis of okara; generally, it consists of 25.4−28.4% protein, 3.8−5.3% carbohydrate, and 9.3−10.9% oil and fat (*3*).

Therefore, okara contains good proteins and valuable components from soybeans, which possess food functions such as anticholesterol action (*5*) and prevention of liver fat accumulation (*6*). Okara is also recognized as an important food fiber (*7*). However, the use of okara has been difficult because of its high fiber content and unsavory texture for eating. Many trials for using okara have been reported; however, most of these reports were on its fermentation (*8*, *9*), extraction of protein from okara, utilization for animal feed or mushroom medium (*10*), and extraction of emulsionizing polysaccharides from okara (*11*). Okara is still an agricultural waste product (*1*).

Some extractions of soluble polysaccharides from soybeans and okara were reported (*12*−*16*). However, studies of the solubilization of okara itself are few and have not been reported to be successful yet. Yamaguchi et al. reported that the lignin of okara was a problem regarding its solubilization, and okara enzymatic solubilization was carried out with a yield of 72.3% using many enzymes such as cellulase, pectinase, and lacchase for 10 days (*17*). Muzakha et al. reported screening to digest okara and the isolation of *Aspergillus niger* var.Tieghe KF-267 as a new okara-liquefying mold. They tried to digest okara with crude enzyme solutions containing α-L-arabinosidase and endo β-D-1,4-galactanase, but their enzymatic digestion yield was only ∼50% in 60 h (*18*).

On the basis of these results, okara is considered to be composed of highly structured fiber, the extensive digestion of which is very difficult, and the solubilization rate is ∼50−70% using many enzymes over a long reaction time.

We considered the results and reports available and assumed that there were the following problems: (1) Okara is considered to be hard to digest due to its composition of advanced and complicated fibers (*11*, *17*, *18*). (2) Generally, mashed okara is considered to be efficient for enzyme reaction and its analysis (*18*).

Okara does not consist of indigestible fibers but is a complex of soybean cells. It is difficult to study what are digestible and/

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or indigestible tissues when using mashed okara. The plant organization or cell is actually composed of advanced and complex structures (*19*, *20*), and okara from soybeans is no exception. We reported that single-cell formation from soybeans was very effective in cellulase digestion and that the primary cell wall was easily digested but that the secondary cell wall remained (*21*). Therefore, our method and results would be useful in digesting okara, if the secondary cell wall can be digested.

In this paper, we tried to adapt these results (*21*) for okara digestion and studied the composition of the secondary cell wall and the selection of effective enzymes for the digestion of the secondary cell wall. We report the result of enzyme selection, and also a series of enzymatic digestions was adapted to okara, considering its structure and characteristics. An enzymatic high digestion was easily achieved in a yield of 83−85% w/w as a result. Information on the enzymatic soluble structure, its localization, and/or digestible components and the indigestible components are also reported.

MATERIALS AND METHODS

Okara and Pectin. Raw okara was supplied from Matsuda Food Co., Inc. (a tofu and soybean milk manufacturing company in Osaka, Japan). The water content was 80.2% w/w. The raw okara was stored at −80 °C. A part of the okara was dried at 40 °C for 2 days, and it was crushed in an electromotive style mill (personal mill, type SCM-40A, Shibata Co., Ltd., Tokyo, Japan). This powder was used only for pretesting of the cellulase digestion of okara. Pectin (from citrus fruit) was purchased from Wako Pure Chemicals (Osaka, Japan) and was washed continuously with 80, 90, and 99% ethanol and finally with acetone and dried for analysis. Okara and its reaction residues were freeze-dried for analysis.

Enzymes. Cellulase (for food processing, 5000 units/g) and glucanase were kindly given by Daiwa-Kasei Co., Ltd. (Osaka, Japan). Pectinase (Pectinex Ultra SP; 26000 units/mL) was kindly given by Novozyme Japan. Protopectinase was supplied from Kurabo Co., Ltd. (Osaka, Japan). Hemicellulase, pectinases, and proteases were kindly given by Amano Enzyme Co., Ltd. (Nagoya, Japan) and Daiwa-Kasei Co., Ltd. (Osaka, Japan). Reagents for pectinases and pectolyase from *Aspergillus japonicus*, pectinase from *Rhizopus* sp., pectin lyase from *A. japonicus*, and pectinase from *Aspergillus niger* were purchased from Sigma Chemical Co., St. Louis, MO. Other reagents were used as reagent grade.

Pretesting of Cellulase Digestion for Okara. Dried and powdered okara (0.5 g) and water (7 mL) were mixed in a 20 mL test tube with or without autoclaving (121 °C), and cellulase was reacted at 40 °C. This examination was done to evaluate the autoclave time, the amount of enzyme, and the reaction time. The hydrolysis was evaluated by the amount of sugar released.

Enzymatic Digestion of Okara. Two grams of raw okara and 13.4 mL of water were placed in a glass vessel (diameter = 30 mm × height = 63 mm), and treatment in an autoclave (121 °C, for 20 min) was carried out. Cellulase (50 units/g of okara) was added, mixed, and then reacted during stirring with a stirrer bar (6 × 25 mm) at 40 °C for 15 h on a magnetic stirrer, model RC-2 (Tokyo Rika Kikai, Tokyo, Japan). The reaction was stopped by boiling at 100 °C for 10 min. The reaction mixture was centrifuged (3000 rpm, for 10 min), and the amounts of sugar and protein released into the supernatant were evaluated. The precipitated residue was collected and washed with water, and the residue was freeze-dried; the dried weight was measured.

Estimation of Sugar and Protein. The amount of uronic acid was measured according to the 3-phenylphenol method (*22*), and the total sugar was estimated by the phenol−sulfuric acid method (*23*). The reducing sugar was estimated according to the Nelson−Somogyi (*24*) method, and protein was estimated by the Lowry method (*25*). Each amount was calculated by a colorimetric standard curve using D-galacturonic acid, D-glucose, and serum albumin, respectively, as standards.

Neutral Sugar Analysis. Analysis of neutral sugars was done using the alditol−acetate method (*26*). The composition of neutral sugar was analyzed by a GC system with a capillary column of DB-225 (J&W Co., 0.25 mm × 30 m) connected to a Yanaco G-2800 (Yanaco Co., Ltd., Kyoto, Japan). The standard solution was adjusted with a 1% solution of each of seven kinds of sugar: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose.

Staining and Observation of Okara. One gram of fast green was dissolved in 100 mL of 7% acetic acid and kept in a sealed dark brown bottle after the filtration. Another staining for protein was done with acrolein−Schiff reagent. Polysaccharides were stained by PAS dyeing (*27*).

Light Microscopic Observation. Microscopic observation and photos were done using a light microscope, Olympus model BH-21 (Olympus Optical Co., Ltd., Tokyo, Japan) and a digital microscope photography device, DP-II.

Preparation of Dissolved Secondary Cell Wall from Okara. Okara residue after autoclaving and cellulase digestion was dissolved in 1 N NaOH with boiling for 10 min. The solution was subjected to preparative gel chromatography. The chromatography was done with a column of TSK gel Toyopearl HW-50 (Toso, Tokyo, Japan). The column (28 × 530 mm) was eluted with water, and the fractionation was 5 mL/fraction.

SDS-PAGE Electrophoresis. SDS-PAGE was done according to the Laemmli method (*28*). The electrophoresis equipment used was an AE-9631M/P with an attached concentration slope gel (5−20%) (manufactured by ATTO, Tokyo, Japan). The standard molecular marker was the Kaleidoscope Prestained Standards from Bio-Rad.

Measurement of the Degree of Esterification. The sample was analyzed in accordance with the method of Keith et al. (*29*) by gas chromatography. Raw okara (5 mg) treated with cellulase and citrus pectin (5 mg) were placed in a test tube with 2 mL of water and stirred well in a vortex mixer. Then 0.8 mL of 2 N NaOH was added, and the solution was held at room temperature for 1 h with stirring. The reaction was stopped and neutralized by the addition of 0.8 mL of 2 N HCl, and the solution was filtered. *n*-Propanol (0.4 mL, 0.2%) was added as a standard. The sample was made up to 4 mL with water and analyzed by GC. The GC analysis was done with a PEG-20M glass-packed column (Uniport HP, 4 mm × 1 m) attached to Yanaco G2800 GC equipment. Analysis conditions were as follows: oven temperature, 45 °C; injection temperature, 100 °C; detector, FID.

HPLC Analysis. The analytical HPLC system DP8020 (Toso, Tokyo, Japan) was used with a difference refraction indicator RI8020 (Toso) and an optical photometer UV8020 (Toso) for detection.

Identification of Uronic Acid. Cellulase-treated raw okara and citrus pectin, each at 20 mg, were crushed and hydrolyzed with 4 N sulfuric acid at 100 °C for 1−3 days. The sample was neutralized with 4 N NaOH and centrifuged. The sample (10 μL) was analyzed by HPLC. HPLC conditions were as follows: column, Shodex Asahipak NH2P-504E, 4.6 × 250 mm (Showa-Denko Co., Ltd., Tokyo, Japan); eluent, 0.3 M phosphoric acid buffer solution (pH 4.4); flow rate, 1 mL/min; column temperature, 40 °C; detector, 210 nm.

Component Analysis of the Final Okara Residue. *Uronic Acid and Sugar.* The final okara residue (dried weight = 10 mg) was hydrolyzed in 1 mL of 2 N HCl at 100 °C for 2.5 h. The hydrolysis mixture was cooled and centrifuged (3000 rpm for 10 min); the uronic acid and total sugars of the supernatant were measured.

Protein. The final okara residue (dried weight = 10 mg) was hydrolyzed in 1 mL of 2 N NaOH at 100 °C for 10 min; soluble protein was measured according to the Lowry method.

Oil. Hexane was added to 100 mg of the final residue and mixed in a vortex mixer for 5 min; the hexane layer was separated and collected by centrifugation (3000 rpm for 10 min). This extraction was done three times, and the product was evaporated and dried in vacuo. The weight of the residual oil was measured. The final residue was directly observed with a light microscope.

RESULTS

Preinvestigation of Okara Digestion Using Cellulase. The effects of autoclaving time, amount of cellulase, and cellulase

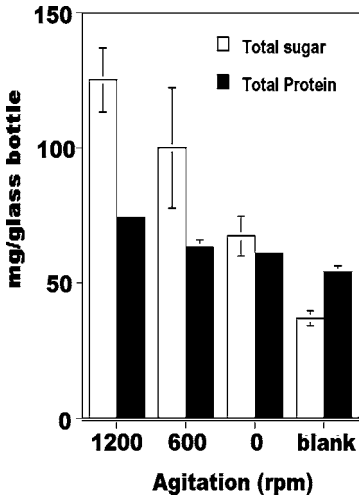

Figure 1. Effect of stirring on okara cellulase digestion: Raw okara (2 g) and water (13.4 mL) were added to a glass bottle and autoclaved at 121 °C for 20 min. The glass bottle was incubated with stirring at 40 °C for 15 h with cellulase (1%). The supernatant of the reaction mixture was analyzed. Each reaction was done three times.

reaction time on okara digestion were investigated using dried and powdered okara.

Effect of Autoclaving Time. The autoclaving was effective in digestion with cellulase, and the conditioning time was sufficient for >10 min at 121 °C; a longer autoclaving was not more effective.

Effect of Amount of Cellulase. Hydrolysis was linearly increased in proportion to cellulase addition within 1%; however, >1% cellulase addition was not effective and an increase in hydrolysates was not recognized.

Effect of Reaction Time. When the autoclaving time was 20 min and 1% the amount of cellulase, the reaction time was sufficient for 15 h. An increase of reducing sugar was detected, but an increase in the total sugar released was not recognized over even 1 week (data are not shown). The cellulase digestible component of okara after the autoclaving treatment was easily and quickly digested by cellulase; however, the cellulase-indigestible component of okara was not digested independent of its reaction time.

Agitation Effect for Okara Cellulase Digestion. Figure 1 shows the effect of agitation. A static hydrolysis reaction using cellulase produced little digestion. Increasing the stirring caused better okara digestion by cellulase. The optimum okara content was 13% (w/v), and the agitation was 1200 rpm. This concentration of okara was the maximal dose for agitation by a magnetic stirrer bar in starting digestion. This agitation was the maximal stirring with our equipment, but greater agitation may provide better digestion.

Light Microscopic Investigation of Cellulase-Digested Okara. Figure 2 shows the light microscopic photos of the effect of autoclaving and agitation on the cellulase digestions of okara. In these digestions, there were few changes treated with only autoclaving (**Figure 2A**). Lack of agitation could not induce the formation of single cells from the okara (**Figure 2B**). Greater agitation accelerated the formation of single cells from the autoclaved okara; the remaining indigestible okara was found to be transparent thin cell walls and fiber-like organ attached to the walls (**Figure 2C**).

Comparison of Remaining Okara and Single Cells of Soybeans after Cellulase Digestion. Comparison of the

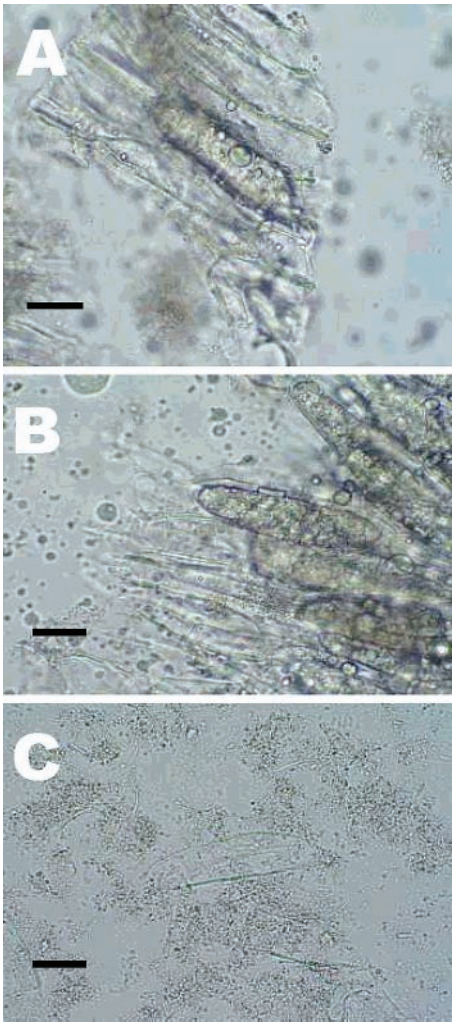

Figure 2. Light microscopy of cellulase-digested okara. Digested okara in a glass bottle was directly observed using light microscopy: (**A**) starting raw okara; (**B**) cellulase digestion of autoclaved okara without stirring; (**C**) cellulase digestion of autoclaved okara with stirring (1200 rpm). Black bar represents 10 μm.

remaining indigestible okara and cellulase-digested single cells of soybeans was carried out. **Figure 3** shows the results. The primary cell walls of the single cells of soybeans and okara were digested and removed (**Figure 3A**). In the soybean single cells, it was recognized that the thin secondary cell wall was swollen and contained inner structured cell bodies (**Figure 3B**) (*21*). Similar cell walls were also found in the okara treated with cellulase digestion. Therefore, the indigestible cell walls from okara cellulase digestion were identified as the secondary cell walls of soybeans. The major part of the oil and protein bodies of soybean cells was removed into the soybean milk in the case of okara, and most of the remaining indigestible okara was considered to be the secondary cell walls of soybeans.

Composition of Remaining Cellulase-Digested Okara. Staining with PAS, fast green, and acrolein−Schiff reagent did not produce clear positive staining for the secondary cell walls; all stainings were pseudo-positive. Alkaline treatment of the remaining okara after the cellulase digestion was done with 1 N NaOH and boiling for 10 min, and the soluble part was subjected to gel filtration on Toyopearl HW-50 and eluted with water. **Figure 4** shows a typical elution profile. Total sugar, uronic acid, and protein were detected as one peak of the high molecular weight fraction. The ratio of neutral sugar and uronic

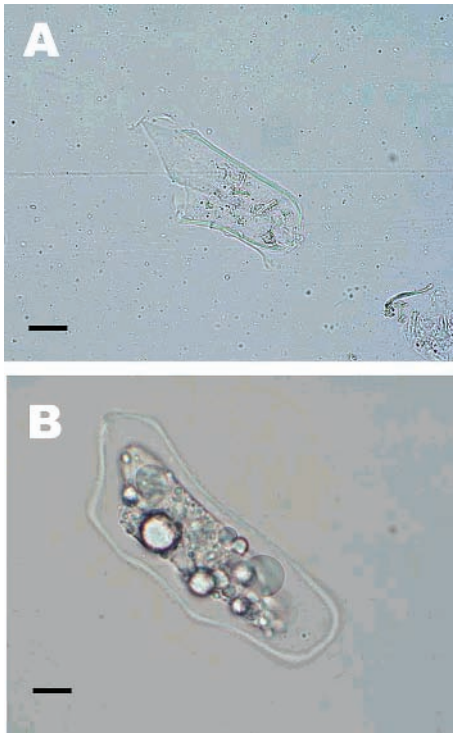

Figure 3. Comparison of the secondary cell walls from okara after treatment by cellulase digestion and single soybean cells after autoclaving and cellulase digestion: (**A**) single cell from okara after autoclaving and cellulase digestion; (**B**) single cell of soybean after autoclaving and cellulase digestion. Each sample was observed by light microscopy. Black bar represents 10 μm.

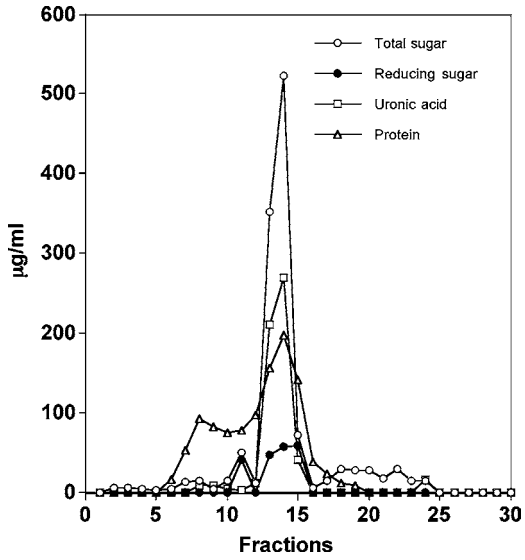

Figure 4. Gel permeation chromatography of alkali-dissolved secondary cell walls: Twenty milligrams of dried okara residue after autoclaving and cellulase treatment was added to 5 mL of 1 N NaOH. The mixture was heated in boiling water for 10 min, and the dissolved sample was applied to a column of Toyopearl HW-50 (28 × 530 mm). The fractionation was 5 mL per fraction.

acid was 1.12:1. SDS-PAGE of the fraction is shown in **Figure 5**. The results also showed that protein and sugar were found to be the same stained broad spot.

Comparison of Remaining Okara and Citrus Pectin. Hydrolysis of the remaining okara and citrus pection was done with 2 N HCl for 3 h. The supernatant was neutralized and

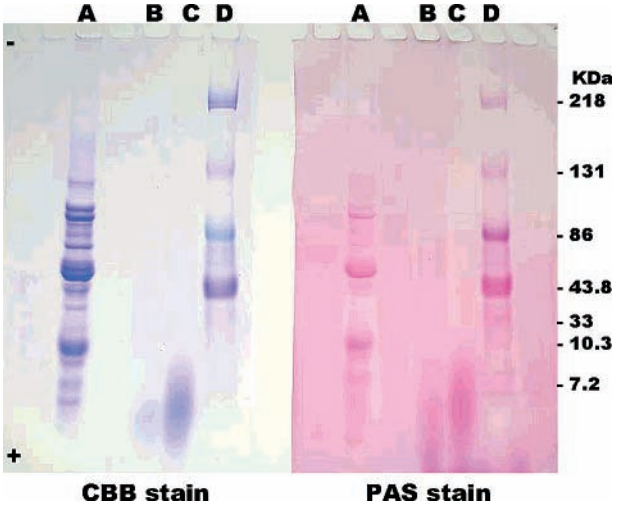

Figure 5. SDS-PAGE of the main fraction in gel chromatography of dissolved secondary cell walls: The main fraction in **Figure 4** was collected and freeze-dried, and then the sample, extracts of soybean meal, and standard makers were analyzed by SDS-PAGE electrophoresis: (lane A) 1% extracts of soybean meal (sample size = 20 μL); (lane B) 1% freeze-dried main fraction from **Figure 4** (sample size = 10 μL); (lane C) same sample of B (sample size = 20 μL); (lane D) standard markers (sample size = 20 μL). Electrophoresis conditions: 20 mA, 90 min. Protein was stained using Coomassie Brilliant Blue (left), and saccharide was stained using PAS staining (right).

applied to an HPLC column. The chromatography profiles were similar, and galacturonic acid was detected. The ester degree of the remaining cellulase-digested okara was determined to be 18.3%.

Screening of Enzymes for the Digestion of Remaining Okara. A number of enzymes for food processing, such as proteases, cellulases, glucanase, hemicellulases, and pectinases, were reacted with the remaining secondary cell walls from okara after cellulase digestion, and the visible changes in the secondary cell walls of okara were monitored by light microscopy. Most of the tested enzymes were not effective in digesting the remaining secondary cell walls from okara. Only two enzymes of Pectinex Ultra SP from *Aspergillus aculeatus* and pectolyase from *A. japonicus* digested the remaining secondary cell walls of okara. Pectinex Ultra SP resulted in good digestion.

Digestion of Remaining Okara by Pectinases of Pectinex. Figure 6 shows the time course of the digestion of the remaining okara after the cellulase digestion by the pectinase of Pectinex Ultra SP. Total sugar, uronic acid, and protein hydolysate were simultaneously released and increased in the same reaction time. The final released amount of protein was higher than the amount of total sugars, although the total sugar and uronic acid release was stopped.

Hydrolysis of Single Cells of Soybeans with Light Microscopy. Figure 7 shows light microscopy photographs before and after enzymatic hydrolysis for the single cells of okara using Pectinex. The secondary cell walls were completely solubilized and disappeared.

Series of Enzymatic Hydrolysis of Okara. Table 1 summarizes a series of digestions of okara. All digestions produced not only sugar and uronic acid but also protein. The reason for this result would be that many parts of the okara are practically composed of these complexes such as the secondary cell walls. The autoclaving was effective in extracting protein. This extract would be the residual of soybean milk and the materials between cells as determined by HPLC analysis and staining results (data

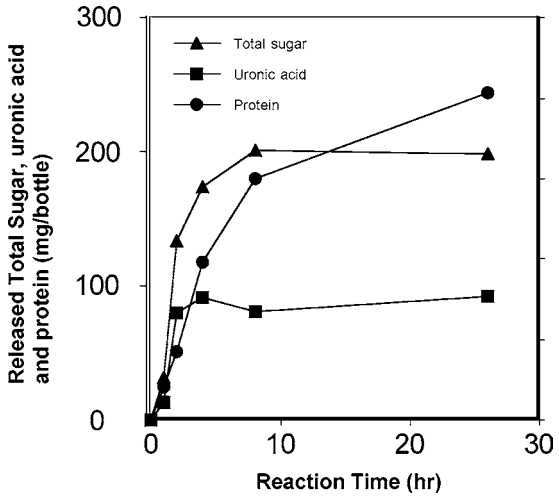

Figure 6. Digestion of okara residue after autoclaving and cellulase treatment using pectinase of Pectinex: The okara residue after treatment with autoclaving and cellulase (0.5 g) was added to 0.1 M acetate buffer, pH 5.0, with the pectinase of Pectinex (100 μL) and was incubated at 40 °C in a glass bottle and stirred with a magnet bar for 24 h. The supernatant of the reaction mixture was analyzed for total sugar, uronic acid, and protein.

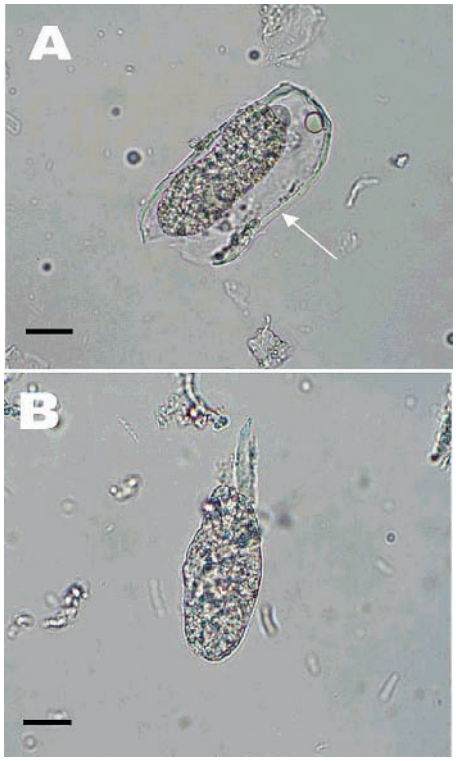

Figure 7. Light microscope photograph of a single soybean cell from okara before and after enzymatic hydrolysis using Pectinex. Cellulase-digested single cells of soybeans of okara and pectinase of Pectinex (1% v/v) were mixed and observed: (**A**) before pectinase reaction, the secondary cell wall (arrow indicated) is found; (**B**) after pectinase reaction for 15 h, the secondary cell wall was digested and disappeared. Black bar represents 10 μm.

are not shown). It is noted that sugar and protein were digested and solubilized as a result, although cellulase digestion was to digest the primary cell walls of okara. The pectinase digestion produced uronic acid; in addition, neutral sugar and protein were also solubilized. **Table 2** shows the analysis of the neutral sugar composition in the series of digestions. The autoclaving treatment produced mainly carbohydrates of glucose and galactose. The cellulase digestion produced half of the neutral sugars as glucose and also xylose and arabinose. The pectinase digestion was remarkable, showing the same quantity of glucose and galactose, and the ratio of arabinose to galactose was 1:1.9. The indigestible residue was rich in xylose, galactose, and glucose; the ratio was 2.6:1.7:1.

Table 1. Component Analysis of a Series of Digestions of Okara[a]

procedure	total sugar (mg)	uronic acid (mg)	protein (mg)	residue (g)
autoclaving	27.9 ± 2.67	8.05 ± 1.60	49.5 ± 12.08	0.323 ± 0.0010
cellulase digestion	45.4 ± 18.60	11.2 ± 0.78	62.1 ± 4.61	0.221 ± 0.0030
pectinase digestion	11.4 ± 5.00	29.14 ± 2.22	15.5 ± 0.71	0.067 ± 0.0076

[a] Samples: $n = 5$; results means ± SD. Raw okara (2 g) and water (13.4 mL) were mixed and then treated and digested by a series of procedures. The samples were centrifuged, and the compositions of each supernatant were analyzed. Each of the residues was collected by centrifugation, and the freeze-dried weight was measured.

Table 2. Molar Ratio of Neutral Sugars in a Series of Digestions and the Final Indigestible Residue of Okara[a]

neutral sugar	autoclaving	cellulase digestion	pectinase digestion	residue
rhamnose	1.1	0.9	2.0	4.2
fucose	0.4	0.0	2.0	0.0
arabinose	0.1	12.5	18.2	0.3
xylose	4.5	20.6	7.7	33.7
mannose	0.0	6.1	0.0	0.0
galactose	32.4	7.8	34.4	22.2
glucose	51.0	52.1	35.6	13.0

[a] Supernatant of okara digestion and the final indigestible residue (**Table 1**) were freeze-dried, and then each sample was hydrolyzed with 2 N HCl at 100 °C for 2 h. The neutral sugar was analyzed according to the alditol–acetate method using GC. Each value is expressed as percent mole.

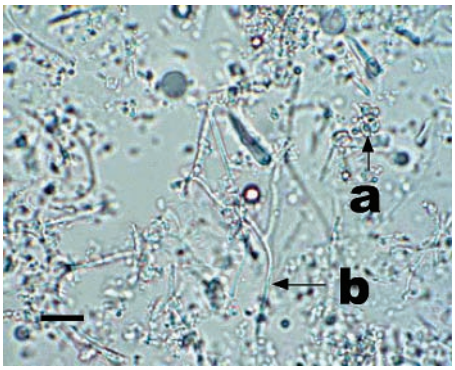

Figure 8. Light microscope photograph of final indigestible okara residues. The final indigestible residue of okara after the series of digestions was observed by light microscopy. Many particles of oil bodies of the inner soybean cells (**a**) and fiber-like organ between cells (**b**) were detected. Black bar represents 10 μm.

The residual okara component was recognized to be oil, oil bodies in the soybeans cells, and fiber-like organs between the cells under light microscopy (**Figure 8**). The chemical components were estimated to be 33.8% (SD = 13.6%) protein, 20.0% (SD = 0.7%) oil, and 22.2% (SD = 5.81%) sugar.

DISCUSSION

Okara comes from soybeans and is the cells of soybean after removal of their ingredients into soybean milk. The cell walls

of the plant cells are considered to contain as main components cellulose, hemicellulose, and pectin, and cellulolytic enzymes are supposed to be easily digestible. However, reports of available and extensive enzymatic digestion were few (*18*), although many water and chemically extractable saccharides of soybeans have been reported (*20*). Therefore, the general understanding is that the indigestible part of okara and/or soybeans is thought to consist of indigestible high and complicated fibers. It is considered difficult to achieve extensive digestion of okara and/or soybeans; however, we achieved a high digestion of okara by clarifying its structure and its characteristics.

In this paper, three important facts were found in digestion. The first was that a single-cell preparation with autoclaving and agitation was effective for digestion by cellulase. The second was that specification and identification of the enzymatically digestible and indigestible parts of okara were achieved. The last was that identification of the components of the indigestible secondary cell walls and a useful pectinase to digest the secondary cell walls were found.

In the first result, cellulase was essentially effective in digesting the primary cell walls, and the digestion became very effective with structure breaking such as by autoclaving and stirring. A large amount of cellulase and a long reaction period were not needed in our results. The cell wall is understood to be mainly composed of cellulose and hemicellulose; however, the real cell wall is protected by several glues, and the cells are attached to each other and are structured (*19*). The raw cell walls of okara are not easily digested by cellulase in real enzymatic digestion. This was also found in the digestion of soybeans for extraction of soybean oil (*21*). Autoclaving was effective for removing the glues between the cells (*21*). Additional agitation was also very effective in cellulase digestion of the primary cell wall, because dispersion of soybean cells of okara was accelerated into single cells, and the naked surface of the soybean cells of okara would increase. Mechanical breaking and enzymatic digestion would produce not only total sugars but also uronic acid and protein in the digestion, although cellulase is a cellulolytic enzyme. The cell wall is not composed of simple cellulose, but the cell wall is structured and complicated with carbohydrates and proteins (*20*). The combination of autoclaving temperature and its period was not investigated in detail, but boiling water would also be effective if the period is long, for example, some hours at least. The cell walls of soybeans boiled for 10 min were hardly digested (*21*).

Second, a light microscopic investigation for comparing the digestion of okara and the soybean single cells was important and helpful. From the microscopic observation, we identified that cellulase-indigestible okara was secondary cell walls, but it was not the status of the cellulase digestion of the cell walls on stopping or during digestion. In the case of beet fiber digestion, the rigid chemical linkage of ferulic acids served as a protection. Williamson et al. reported and showed that ferulic acid esterase is important for digesting beet fibers (*30*). Yamaguchi et al. described that the existence of lignin in okara was a hindrance for its digestion (*17*), and they used lacchase for the digestion, but we could digest okara in high yield without ferulic acid esterase and laccase. The general chemical analysis of okara has been well reported, and the analysis was well done on the total composition (*1*); however, the location and origin of the protein or saccharides were not clear. The origin of residual soybean milk and/or the remaining proteins and saccharides in the cell walls and cells of okara is unknown; this information would be useful for better digestion.

Third, the composition of the cellulase-resistant secondary cell walls was insoluble pectin like material composed of galacturonic acid, neutral sugars, and protein. Electrophoresis of SDS-PAGE and gel filtration chromatography showed that saccharide and protein coexisted, although staining of the secondary cell walls could not provide a clear result. This result would show that these components would be structured and intricately involved with each other; this would be the reason many tested pectinases could not digest the cell walls. Although many studies on soluble pectin from soybeans are known, there are few on insoluble pectin (*31*−*35*). Especially, studies of site identification and composition are few. This secondary cell wall is interesting from the point of its resistance to hemicellulase, proteases, and even many of the pectinases. Chemical analysis showed that the secondary cell wall may be fundamentally similar to a kind of pectin. As a result, we found that two kinds of pectinases for food processing could provide effective digestion.

Protopectin is defined as water insoluble pectin, and various types exist (*35*). Whether the secondary cell wall of okara is a so-called protopectin is not simple. We could not determine which material resists and intercepts enzymes—protein, hemicellulose, steric hindrance, the complex structure or all of the components—and we did not try to use various combinations of proteases and cellulolytic enzymes. Further studies of the compositions and enzymatic studies will be needed. We will study the cellulase- and pectinase-digested fractions in this experiment in detail soon.

The final residues of okara in our experiment were found to be oil body structures (*37*) and fiber-like organ at the boundary between cells in the light microscopic photographs. Composition analysis showed the results were agreed. This result means that some parts of the cells were not broken in manufacturing soybean milk (*38*). Therefore, if a perfect grinding extraction for soybean milk is possible, more okara would be digestible by our method. The ratio of neutral sugar in each digestion and the residue would suggest a typical sugar structure of plants (*20*). The results of cellulase digestion would suggest cellulose and hemicellulose. The results of pectinase digestion, the secondary cell wall of pectin-like material, would indicate the arabino-galactan component (ara:gal = 1:2) of pectin (*16*, *20*). The xylose could come from xylo-galacturonan as characteristic of legumes (*14*, *39*). The existence of glucose was not accounted for in our examination. The neutral sugar of the residue was remarkable; the main sugar was xylose. The ratio of xylose to galactose to glucose was about 3:2:1; this ratio might be a regular structure. An additional remarkable result was that neutral sugar, uronic acid, and protein were detected in the series of digestions. Digestion by cellulolytic enzymes produced not just those specific saccharides. The residual oil and protein body complexes are composed of protein and saccharides, and the residual fiber-like organ would be composed of protein and cellulose-like material (unpublished data). As for the oil body, Tzen et al. already reported that trypsin is effective in digesting purified oil bodies and that the oil bodies consisted of oil (~95%) (*40*). However, digestion of raw oil bodies and the whole structured complex has not been reported; we have already obtained results in which oil body complexes of soybean are resistant to trypsin (*21*). We are now trying to develop a perfect digestion of the final residual okara and soybeans using various enzymes.

Soybeans are widely used as a foodstuff, and their agricultural waste is a problem. For example, another typical waste from soybeans is soy sauce residue, which is an agricultural waste

from soybeans as well as okara; the effective utilization has been considered to be very difficult. It is reported that enzymatic extraction of defatted soybeans is also incomplete (*41*); however, these are principally soybeans, so we think that our method could be usable and helpful in digesting defatted soybeans. There are diverse plant cell wall structures, so that further enzymatic stripping and digestion should be studied. Our results will be useful for a method of digestion and/or processing of other agricultural products and their waste.

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